EGFR-AKT-SMAD signaling promotes formation of glioma stem-like cells and tumor angiogenesis by ID3-driven cytokine induction

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Abstract

Aberrant activation of receptor tyrosine kinases is causally linked to the pathobiological traits of glioblastoma and genesis of glioma stem-like cells (GSCs), but the underlying mechanism is still unknown. Here we show that epidermal growth factor receptor (EGFR) signaling regulates the proliferation, angiogenesis and acquisition of GSC characteristics by inducing inhibitor of differentiation 3 (ID3) and ID3-regulated cytokines (GRO1, IL6, and IL8) induction. We found that EGFR-mediated ID3 expression was regulated by Smad5, which was directly phosphorylated by AKT. Furthermore, ID3 alone imparted GSC features to primary astrocytes derived from Ink4a/Arf-deficient mouse, and EGFR-ID3-IL6 signaling axis gave rise to tumor cell heterogeneity. Conversely, EGFR inhibitors suppressed EGFR-AKT-Smad5-driven induction of ID3, which led to a decrease in the tumorsphere-forming ability of GSCs and U87MG cells that possess an active mutant EGFR, EGFRvIII, without obvious cytotoxic effects. However, these cells appeared to regain colonogenic ability after removal of the EGFR inhibitors. Together, the results delineate a novel integrative molecular mechanism in which the RTK-ID signaling pathway governs genesis and maintenance of GBM histopathologic features, such as GSCs-based tumor initiation, progression, and angiogenesis.
Introduction

Glioblastoma multiforme (GBM) is one of the most aggressive malignancies that arise from the central nervous system. Despite intensive research over several decades, the median survival of patient with GBM is still less than 15 months (1). GBM is a histologically heterogeneous tumor characterized by high proliferation, anaplastic changes, angiogenesis, invasion, and focal necrosis (1).

One of the most common signaling changes in GBM is the aberrant activation of various receptor tyrosine kinases (RTKs) (2). In particular, the epidermal growth factor receptor (EGFR) is activated in over 50% of patients with GBM through a constitutively active mutation (an in-frame deletion of exons 2–7 of EGFR, referred to here as EGFRvIII) or gene amplification (3). Constitutively activated EGFR signaling confers increased tumorigenicity to glioma cells in vitro and in vivo by increasing proliferation and resistance to apoptosis (4); however, the precise mechanism by which this aberrant EGFR signaling leads to the histopathologic traits of GBM is still unknown.

Some tumors, including GBM, possess a subset of cancer cells (referred to as cancer stem cells, CSCs) that are capable of asymmetrical self-renewal, tumor initiation, and multi-lineage differentiation (5). In particular, CSCs derived from GBM (referred to as glioma stem cells, GSCs) drive tumor growth, invasion, and recurrence after surgical resection, irradiation, and chemotherapy. EGF and bFGF signaling maintain the stemness properties of neural stem cells (NSCs) and GSCs, which give rise to tumors closely resembling the histopathologic phenotype of primary tumors (6).
Inhibitor of the differentiation (ID) family (ID1-ID4) is a group of basic helix-loop-helix (bHLH) proteins that lack a DNA-binding domain and they inhibit the DNA-binding activity of bHLH transcription factors through heterodimerization (7). ID proteins, which are cell fate determinants, are involved in a broad range of processes associated with tumorigenesis. For example, ID proteins promote cell proliferation by suppressing cell cycle negative regulators (8); and ID1 and ID3 are required for tumor angiogenesis and secondary breast tumor re-initiation in lung metastasis (9, 10). We and others have recently demonstrated that ID4 induces brain cancer stem cell genesis through dedifferentiation of Ink4a/Arf-deficient mouse astrocytes (11), and that ID1 and ID3 induced by TGF-β signaling mediate the tumor-initiating capacity of GSCs (12).

These findings suggest that there is a crucial link between RTKs and the ID family in glioma biology; however, little is known about the signaling responsible for GSC genesis and maintenance. In the present study, we show that EGFR signaling leads to acquisition of GSC characteristics and angiogenesis by induction of ID3 and ID3-regulated cytokines through AKT-dependent activation of Smad5.
Materials and Methods

Cell lines, cell growth, neurosphere formation, and colony formation on semisolid medium

The human glioma cell lines (U87MG, A1207, and U373MG) and primary human umbilical vein endothelial cells (HUVECs) were purchased from American Type Culture Collection (ATCC) and were maintained for fewer than 6 months after receipt. These cell lines were authenticated by ATCC and through various tests. Mouse astrocytes were isolated from the cerebral cortices of 5-day-old Ink4a/Arf-knockout mice as described previously (13). X01, X02 and X03 GSC lines are glioma stem cell lines established from human brain tumors (14). Cell growth and neurosphere formation assay was performed as described previously (11). Colony formation on semisolid medium was performed as described early (15).

Subcutaneous and orthotopic implantation assay

To establish subcutaneous xenograft models, cells (2×10^6) were subcutaneously injected into nude mice (BALB/c nu/nu mice). For the orthotopic implantation, 5×10^4 cells were stereotactically injected into the brain of nude mice (coordinates: anterior-posterior, +2.5; medial-lateral, +2; dorsal-ventral, −3 mm from the bregma). All mouse experiments were approved by the animal care committee at the College of Life Science and Biotechnology, Korea University, and were performed in accordance with government and institutional guidelines and regulations.

Statistics

For the multi-dataset experiments, ANOVA tests were performed using the Statistical Package for the Social Sciences software (version 12.0; SPSS, Chigaco). a,b,c,d P < 0.05 or A,B,C,D P < 0.01 was considered to represent a statistically significant difference. For the two-dataset experiments,
two-tailed Student’s t-test was performed. *P < 0.05 or **P < 0.01 was considered statistically significant.
Results

EGFR-AKT-Smad1,5,8 signaling axis induces ID3 in human glioma cells and GSCs

Aberrant activation of EGFR signaling is causally linked to gliomagenesis (2) and constitutively activated EGFR signaling imparts stem cell-like characteristics in Ink4a/Arf−/− mouse astrocytes (13). However, the underlying mechanism of EGFR-driven acquisition of GSC features is still unknown. Glioma stem cells (GSCs) share a number of traits with normal neural stem cells, which are governed by stem cell fate determinants. In this study, we focused on inhibitor of the differentiation (ID) family (ID1-ID4) to evaluate their roles in EGFR-driven acquisition of GSC properties. We first determined the expression of four ID genes in many types of human brain malignancies using the mining REMBRANT (Repository of Molecular Brain Neoplasia Data) database (http://caintegrator-info.nci.nih.gov/rembrant). Mean expression levels of ID3 were more than 7-fold higher in astrocytomas (n = 148), GBMs (n = 226), and mixed tumors (n = 11) than normal brain tissue (n = 28) (Supplementary Fig. S1A). We also found that ID3 mRNA levels were increased in 12 of 15 GBM specimens (Supplementary Fig. S1B). These data suggest that ID3 is the most relevant of the four ID genes in glioma pathogenesis. We then compared the expression of four ID genes with EGFR expression in human gliomas using a web-based bioinformatics database obtained from The Cancer Genome Atlas database of National Cancer Institute (TCGA). Of the four ID genes, ID3 mRNA expression showed the strongest association with EGFR genomic amplification (80%; ID3 mRNA increased in 53 of 66 EGFR-amplified glioma specimens) and overexpression (98%; in 164 of 168 EGFR-upregulated glioma specimens) (Supplementary Fig. S2A). To determine whether EGF signaling is directly associated with ID3 expression, three glioma cells, A1207, U373MG, and U87MG, were treated with EGF. EGF treatment consistently increased ID3 expression in three glioma cells with increases in AKT and ERK phosphorylation.
(Supplementary Fig. S2B). We also measured ID3 expression in U87MG cells transduced with two different types of constitutively activated EGFR mutants (EGFRvIII, common in human glioma; and EGFRL858R, common in human lung cancer) and wild-type EGFR, and found that ID3 protein levels were elevated by both mutant and wild-type EGFR genes (Fig. 1A). In a previous study, ID1 and ID3 transcription was shown to be upregulated by phosphorylated Smad1,5,8 (p-Smad1,5,8) (16); therefore, we used a luciferase reporter assay with the ID3 promoter construct (containing a Smad-binding element). EGF promotes transcription of the ID3 promoter in a dose dependent manner, but this effect was completely suppressed by dorsomorphin, a chemical inhibitor of p-Smad1,5,8 (17) (Supplementary Fig. S2C). We also found that ectopic expression of EGFRvIII in U87MG cells increased p-Smad1,5,8, and conversely, dorsomorphin completely diminished ID3 and p-Smad1,5,8 protein and mRNA expression (Fig. 1B upper panel; and Supplementary Fig. S2D), suggesting that EGFRvIII regulates ID3 expression through p-Smad1,5,8. However, previous studies have shown that BMP induces ID expression through BMP receptor (BMPR)-mediated phosphorylation of Smad1,5,8 (16) and induces differentiation of GSCs to non-tumorigenic differentiated glioma cells (18). To determine whether EGF signaling requires BMP/BMPR signaling to induce ID3 and p-Smad1,5,8, we treated cells with the BMP antagonist noggin (19), which strongly reduced p-Smad1,5,8 levels in U87MG-Puro control cells, but did not completely deplete p-Smad1,5,8 levels in U87MG-EGFRvIII cells (Fig.1B lower panel). Furthermore, although ID3 expression was weakly reduced by noggin, EGFRvIII overexpression effectively preserved ID3 expression during noggin treatment (Fig. 1B lower panel), indicating that EGFRvIII might regulate ID3 expression and Smad1,5,8 phosphorylation in a BMP/BMPR signaling-independent manner. To evaluate the plausible mechanism underlying EGFRvIII-driven induction of ID3 and p-Smad1,5,8, AKT and ERK signaling in EGFRvIII-overexpressing U87MG cells was
inhibited with LY294002 and U0126, respectively. Inhibition of AKT, but not ERK, reduced p-Smad1,5,8 and ID3 levels (Fig. 1C), and conversely, ectopic expression of myristoylated AKT (myr-AKT; a constitutively active AKT) in U87MG cells increased p-Smad1,5,8 and ID3 levels (Fig. 1D). These results indicated that EGFR-AKT signaling regulates ID3 expression through p-Smad1,5,8.

**AKT phosphorylates Smad5 and subsequently induces ID3 expression**

To determine how EGFR-AKT signaling induces p-Smad1,5,8, a co-immunoprecipitation assay was performed and Flag-tagged myr-AKT was shown to specifically bind to endogenous Smad1,5,8 (Fig. 2A left panel). A previous study showed that Smad1 and Smad5 played distinct and even opposite roles in embryogenesis (20); thus, the expression levels of each Smad1,5,8 was measured by Q-RT-PCR. In this analysis, Smad5 was found to be the most abundant Smad in U87MG-EGFRvIII cells (Supplementary Fig. S3A). Each Smad1,5,8 was depleted using a siRNA-mediated knockdown method. Although all three Smad1,5,8 mRNA levels decreased by over 50% in U87MG-EGFRvIII cells transduced with each Smad1,5,8-specific siRNA, both total and phosphorylated Smad1,5,8 and ID3 protein levels only decreased in the cells transduced with Smad5-specific siRNA (Fig. 2A middle panel; and Supplementary Fig. S3B), suggesting that Smad5 might be a major regulator of ID3 in EGFR-AKT signaling axis in U87MG cells. To evaluate whether AKT directly induces Smad5 phosphorylation, an in vitro kinase assay was performed and an active myr-AKT, but not a dominant negative AKT mutant (K179M), was shown to enable phosphorylation of GST-Smad5 (Fig. 2A right panel). We also found that AKT-mediated phosphorylation of Smad5 was specific, due to failure in phosphorylation of GST-Smad1 by myr-AKT (Fig. 2A right panel). Collectively, these findings indicate that EGFRvIII-AKT signaling regulates Smad5 phosphorylation and subsequent ID3 expression.
To further characterize the EGFR-ID3 signaling pathway in GSC biology, three different GSCs, X01, X02 and X03 (14), were used. These GSCs exhibit higher levels of ID3 and p-AKT because X01 and X03 express the mutant EGFRvIII and X02 is PTEN-deficient (Fig. 2B upper panel). Consistent with results obtained with glioma cells, LY294002 reduced levels of p-AKT, p-Smad1,5,8, and ID3 in X01 and X02 cells (Fig. 2B lower panel). The shRNA-mediated knockdown of ID3 in these GSCs showed a significant decrease in the neurosphere forming ability, which is a hallmark of GSCs (Fig. 2C). Furthermore, inhibition of p-Smad1,5,8, and the concomitant decrease of ID3 in X01 and X03 by dorsomorphin completely suppressed neurosphere formation (Fig. 2D). These findings indicate that ID3 and p-Smad1,5,8 play a pivotal role in GSC maintenance.

**ID3 imparts glioma stem cell-like features to Ink4a/Arf-/- mouse astrocytes**

To directly address whether ID3 alone is sufficient to give rise to glioma stem-like cells, ID3 was introduced into primary Ink4a/Arf-/- mouse astrocytes by retroviral transduction. ID3 overexpression accelerated cell proliferation with marked increase in cyclin E protein levels, which led to cell aggregation in culture medium supplemented with 10% and 0.5% FBS (Fig. 3A), and produced significant increases in *in vitro* colony formation on a semisolid medium and *in vivo* tumor formation by subcutaneous implantation (Fig. 3B). Our previous study demonstrated that ectopic expression of ID4 in Ink4a/Arf-/- mouse astrocytes led to glioma stem cell genesis (11); thus, we investigated the potential role of ID3 in neurosphere formation and stem cell marker expression. When cultured in serum-free medium supplemented with EGF and bFGF, ID3-overexpressing cells formed neurospheres expressing a number of GSC markers CD133, Nestin, and Sox2, but not the differentiated cell lineage markers S100β (astrocyte), Tuj1 (neuron), and O4 (oligodendrocyte) (Fig. 3C). When cultured in the presence of serum, ID3-overexpressing cells grown in adherent culture showed increased Nestin and Sox2 levels and decreased GFAP levels
(Supplementary Fig. S4), indicating ID3 overexpression induced dedifferentiation of Ink4a/Arf-/- mouse astrocytes.

Given that GSCs are causally linked to GBM heterogeneity, we assessed whether tumors arising from Ink4a/Arf-/- astrocytes-ID3 cells retain their histopathologic features by immunostaining tumor sections with antibodies against neural stem cell and differentiated cell markers. In sharp contrast to control tumors consisting primarily of S100β+ astrocytes, ID3-driven tumors retained CD133+, Nestin+, S100β+, and O4+ cells, as well as micro-and macro-vasculature structures surrounded by CD31+ endothelial cells (Fig. 3D). These results indicate that ectopic expression of ID3 is sufficient to give rise to histologically heterogeneous gliomas with angiogenesis.

**ID3 regulates angiogenesis and GSC genesis by cytokine induction**

Glioma angiogenesis provides a perivasculature niche that allows GSCs to maintain their stemness (21, 22). To determine a possible role of ID3 in angiogenesis (23) and tumor cell heterogeneity, cytokine/chemokine secretion was measured using a custom angiogenesis cytokine antibody array in U87MG-ID3 and control cells, and three cytokines were shown to be upregulated (both expression and secretion) by ID3 overexpression; growth-regulated alpha protein (GRO1, also known as CXCL1), interleukin 6 (IL6), and interleukin 8 (IL8) (Fig. 4A). To evaluate their angiogenic effects, human umbilical vein endothelial cells (HUVECs) were exposed to conditioned medium (CM) from U87MG cells overexpressing ID3 (U87MG-ID3), GRO1, IL6, and IL8 or U87MG-ID3 cells deficient in GRO1, IL6, and IL8 by a short-hairpin RNA (shRNA)-mediated knockdown. We found that HUVEC in vitro vessel formation was markedly increased after exposure to conditioned medium from U87MG cells overexpressing GRO1, IL6, IL8, and ID3 (Fig. 4B). Conversely, depletion of GRO1, IL6, and IL8 in the
U87MG-ID3 cells suppressed vessel formation (Fig. 4B), indicating that ID3-driven angiogenesis in vitro and in vivo (Fig. 3D) is regulated by angiogenic cytokines. In addition, it is worth noting that although EGF is a weaker angiogenic factor than bFGF when added directly to HUVECs; however, the CM derived from U87MG cells treated with EGF led to higher in vitro tube formation of HUVECs when compared to direct treatment of EGF to HUVECs, presumably due to EGFR-ID3 signaling-induced cytokines (Supplementary Fig. S5A). To address this possibility, in vitro tube formation of HUVECs were compared using CM from U87MG-EGFRvIII cells treated with Gefitinib (EGFR inhibitor), LY294002 (PI3K/AKT inhibitor), and dorsomorphin (Smad1,5,8 inhibitor). In contrast to CM from U87MG-EGFRvIII cells, in vitro tube formation of HUVEC was dramatically decreased by CM from U87MG-EGFRvIII cells treated with EGFR, AKT, and Smad inhibitors (Supplementary Fig. S5B). These results suggest that EGF might play a more important role in angiogenesis through EGFR-ID3 signaling-mediated induction of angiogenic cytokines in tumor cells.

We next assessed the neurosphere-forming ability of U87MG cells to determine the biological effect of these genes in GSC genesis. Overexpression of ID3, IL6, IL8, and EGFRvIII (U87MG-EGFRvIII), but not GRO1, promoted neurosphere formation, and depletion of IL6 and IL8 in U87MG-ID3 cells or knockdown of ID3 in U87MG-EGFRvIII cells inhibited neurosphere formation (Fig. 4C). Similar to previous studies, which reported that EGFR-driven overexpression of IL6 promotes breast CSC formation through STAT3-mediated activation of Jagged-Notch signaling (24), we found that mRNA levels of genes related to Jagged-Notch signaling (JAG1, Hes1, and Hey1) and neural stem cell markers (CD133 and Nestin) significantly increased with ID3 and IL6 overexpression. However, IL8 overexpression only weakly induced Hey1, CD133, and Nestin expression, and GRO1 overexpression increased only
Hey1 mRNA levels (Fig. 5A). We also found that shRNA-mediated knockdown of ID3 in the X01 and X02 GSC lines decreased CD133, Nestin, IL6, IL8, Jagged1, Hes1, and Hey1 expression (Fig. 5B). These findings provide evidence that EGFRvIII and EGFR-driven ID3 induction in glioma cells confers GSC features, at least in part, through IL6-Jagged-Notch signaling axis.

**EGFR-ID3-IL6 signaling axis regulates tumor cell heterogeneity**

We then focused on the biological function of the EGFR-ID3-IL6 signaling pathway in GSC-driven tumor progression and heterogeneity. An enzyme-linked immunosorbent assay (ELISA) was used to measure IL6 secretion in U87MG cells treated with EGF and bFGF, and in U87MG cells overexpressing ID3, H-Ras, EGFRL858R, and EGFRvIII. In agreement with previous reports (25, 26), EGFRL858R, H-Ras, and bFGF increased IL6 secretion, as did EGF treatment and overexpression of ID3 and EGFRvIII (Supplementary Fig. S6A). To further assess whether IL6 expression and secretion were dependent on ID3-upstream signaling pathway, U87MG-EGFRvIII cells were treated with Gefitinib, LY294002, and dorsomorphin to inhibit EGFR-AKT-Smad signaling pathway. We found that these inhibitors significantly suppressed IL6 expression and secretion (Supplementary Fig. S6A and S6B). A previous study showed that activated STAT3 increased IL6 expression in an autocrine manner. Therefore, IL6 expression in U87MG-EGFRvIII cells was examined after inhibiting ID3 and JAK/STAT (27). We found that shRNA-mediated ID3 depletion, JAK inhibitor (P6), and STAT3 inhibitor (STAT3-I, an inhibitory peptide against STAT3 dimerization) decreased IL6 mRNA expression by 96%, 24%, and 16%, respectively (Supplementary Fig S6C), indicating that ID3 is a major regulator of EGFR-driven IL6 expression. However, because ID3 is a transcriptional repressor (7), these observations also raise the question of how does ID3 promotes IL6 expression. We found that
ID3 overexpression inhibits transcription of the IL6 transcriptional repressor BCL6 (28), which contains an ID3-repressive element (E-box) in its own promoter, as evidenced by suppression of BCL6-promoter luciferase activity (Supplementary Fig. S6D).

To investigate the effect of the EGFR-ID3-IL6 signaling axis on tumorigenicity and heterogeneity, we injected the following cells into immunocompromised mice: U87MG-IL6, U87MG-EGFRvIII, U87MG-ID3, ID3-depleted U87MG-EGFRvIII, and IL6-depleted U87MG-ID3. In these experiments, we found that EGFRvIII, ID3, and IL6 accelerated tumor formation, while suppression of ID3 and IL6 in the U87MG-EGFRvIII and U87MG-ID3, respectively, significantly reduced (Fig. 6A). As shown in Fig. 6B, cells in tumors derived from U87MG overexpressing IL6, EGFRvIII, and ID3 were positive for neural stem cell markers (CD15 and Nestin), oligodendrocytes (NG2), neurons (Tuj1), and astrocytes (S100β), as well as endothelial cell markers (CD31), demonstrating tumor cell heterogeneity. In sharp contrast, most cells comprising tumors derived from ID3-depleted U87MG-EGFRvIII and IL6-depleted U87MG-ID3 cell were S100β+ astrocytes, implying that these tumors were homogenous, which is rarely observed in human GBM specimens. To assess whether the EGFR-ID3-IL6 signaling axis affects survival of brain tumor-bearing animals, we orthotopically injected these cells into nude mice. Overexpression of EGFRvIII, ID3 and IL6 significantly shortened the survival of these tumor-bearing mice, and conversely, depletion of ID3 and IL6 in the U87MG-EGFRvIII and U87MG-ID3, respectively, dramatically delayed mice death (Fig. 6C). These combined findings indicate that EGF signaling promotes tumor progression and heterogeneity through ID3-IL6 signaling pathway.

Taken together, our results suggest that activation of EGF signaling by genetic alteration (e.g., EGFRvIII) promotes malignant tumor formation and progression through pAKT-pSmad5
signaling-driven ID3 induction and subsequent activation of ID3-regulated cytokines, GRO1, IL6, and IL8.

**Effect of EGFR inhibitors on GSCs with active EGFR-ID3 signaling axis**

We compared the expression pattern of genes associated with the EGFR-ID3 signaling axis in X01 and X03 GSCs during and after treatment with AG1478, which is an EGFR inhibitor. As shown in Fig. 7A, p-EGFR, p-AKT, p-Smad1,5,8, and ID3 protein levels were markedly reduced by AG1478, and withdrawal of this inhibitor led to reactivation of the signaling pathway. Accordingly, treatment of X01, X03, and U87MG-EGFRvIII cells with AG1478 or Gefitinib (another EGFR inhibitor) inhibited neurosphere formation without any obvious cytotoxic effects (Supplementary Fig. S7); however, all cells tested appeared to regain colonogenic ability 12 day after removal of EGFR inhibitors (Fig. 7B), suggesting that continuous EGFR inhibition may be required to suppress tumor progression and recurrence.
Discussion

In this study, we demonstrate that ID3 overexpression alone converts primary Ink4a/Arf-/- astrocytes to cancer cells with GSC features, and EGFR-driven ID3 induction primes GSC genesis by upregulation of IL6. Previous studies have demonstrated that constitutively active EGFR signaling confers stemness features to breast and lung cancer cells by activating Jagged-Notch signaling through IL6-mediated JAK/STAT signaling (24, 26). Similarly, our findings indicate that ID3 switches glioma cells to GSCs with increased IL6 and Notch activity, as evidenced by the induction of Jagged1, Hey1 and Hes1; conversely, depletion of ID3 in EGFRvIII-possessing GSCs reduces IL6, Jagged, Hey1, and Hes1 expression and results in a loss of GSC properties. Under in vivo conditions, GSC maintenance requires specific microenvironments such as a perivascular niche (21). Perivascular nitric oxide or vascular niche factor-PEDF plays important roles of GSC or NSCs maintenance through activation of Notch signal pathway (29, 30). Thus, it is likely that ID3-regulated cytokines (GRO1, IL6 and IL8) play a crucial role in reprogramming of tumor microenvironment that is required for maintenance of GSC traits (Fig. 7C).

A recent study has also demonstrated that IL6 and leukemia inhibitory factor (LIF) expressed from glioma cell lines and glioma stem cells that express EGFRvIII enhance EGFR signaling and proliferation of wild-type EGFR-possessing neighboring cells through gp130-EGFR interaction (31). However, the mechanism of how aberrant EGFR signaling activates IL6 expression is still unknown. However, our findings indicate that ID3 is a crucial intermediate between RTK and cytokine signaling in GSC biology, and that ID3 and IL6 may be potential intracellular and extracellular targets for therapeutic modalities against CSCs.
Previous studies have reported a plausible mechanism underlying the regulation of Smad1,5,8 by the EGFR-AKT signaling pathways. For example, p-AKT inhibits GSK3 activity through phosphorylation (32); GSK3-dependent phosphorylation of the Smad1 linker domain is required for polyubiquitination and subsequent proteasomal degradation of Smad1 (33, 34). However, our data demonstrate that EGFR signaling induces ID3 expression through AKT-dependent induction of p-Smad5, but not p-Smad1. Furthermore, this EGFR-AKT-driven phosphorylation of Smad5 is independent of BMP signaling, which is known to inhibit tumorigenicity of brain tumors (18), because p-Smad1,5,8 levels were maintained in EGFRvIII-overexpressing cells exposed to the BMP antagonist noggin. Although EGFR activation by overexpression or mutation is a common oncogenic event in more than 50% of patients with GBM, only 10% to 20% of these patients responded to EGFR inhibitors in a clinical trial (35). However, the molecular mechanism underlying the resistance to EGFR inhibitors in GBM remains poorly understood. Although pharmaceutical inhibitors of EGFR (AG1478 and Gefitinib) successfully suppress AKT and Smad1,5,8 phosphorylation, ID3 expression, and neurosphere formation in GSCs, these suppressive effects of EGFR inhibitors rapidly disappear after withdrawal of these inhibitors, resulting in recovery of their initial GSC properties. Thus, our study may provide a plausible explanation for tumor recurrence that occurs after treatment with Gefitinib and Erlotinib.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


Figure Legends

Fig. 1. EGFR signaling induces expression of ID3.

(A) ID3 protein level was elevated in U87MG cells transduced with constitutively activated EGFR mutants (EGFRvIII or EGFRL858R) and wild-type EGFR. The protein levels were quantified using Image J software (right panel).

(B) Upper panel: EGFRvIII increased ID3 protein expression in U87MG cells by activating Smad1,5,8 (C-terminal phosphorylation). p-Smad1,5,8 in U87MG-Control and U87MG-EGFRvIII cells was inhibited by dorsomorphin (5 μM, 4 hr). Lower panel: EGFRvIII-driven ID3 induction was not suppressed by noggin (50 ng/ml, 4hr).

(C) AKT activity in U87MG-EGFRvIII cells was specifically required for p-Smad1,5,8-mediated ID3 expression, as assessed by treatment with the MEK/ERK inhibitor, U0126 (U: 20 μM), and the PI3K/AKT kinase inhibitor, LY294002 (LY: 20 μM).

(D) Ectopic expression of myr-AKT in U87MG cells activated Smad1,5,8 and induced ID3 expression.

Fig. 2. AKT directly regulates Smad1,5,8 activity that involves GSC maintenance.

(A) Left panel: Immunoprecipitation assay revealed that Flag-tagged myr-AKT interacts with Smad1,5,8. Middle panel: A siRNA-mediated knockdown of Smad5 decreased ID3 expression. Right panel: In vitro kinase assay revealed that Flag-tagged myr-AKT specifically phosphorylates recombinant GST-Smad5.
(B) Upper panel: Levels of EGFRvIII, PTEN, and ID3 in three GSC lines derived from patients with glioma. NHA, human astrocytes. Lower panel: Inhibition of AKT activity in X01 and X02 by LY294002 decreased p-Smad1,5,8 and ID3 levels.

(C) The shRNA-mediated knockdown of ID3 decreases neurosphere formation in X01 and X02 cells (200 cells/12-well plate). Data are expressed as mean ± SD. *P<0.05; **P<0.01

(D) Inhibition of p-Smad1,5,8 in X01 and X03 cells by dorsomorphin suppressed ID3 expression and neurosphere formation. Data are expressed as mean ± SD. **P<0.01.

**Fig. 3. ID3 switches Ink4a/Arf-deficient mouse astrocytes to GSCs.**

(A) Left panel: Cell proliferation of Ast^{Ink4a/Arf-/-}-control and Ast^{Ink4a/Arf-/-}-ID3 cells grown in DMEM + 10% FBS. Data are expressed as mean ± SD. Inset: representative images of cell grown to confluence in DMEM supplemented with 10% FBS or 0.5% FBS. Scale bar = 20 μm

Right panel: Protein expression levels of cell cycle regulators in Ast^{Ink4a/Arf-/-}-control and Ast^{Ink4a/Arf-/-}-ID3 cells.

(B) Upper panel: Number of transformed foci of Ast^{Ink4a/Arf-/-}-control and Ast^{Ink4a/Arf-/-}-ID3 cells grown on semisolid medium. Data are expressed as mean ± SD. **P<0.01. Right panel: Tumorigenic potential of Ast^{Ink4a/Arf-/-}-control and Ast^{Ink4a/Arf-/-}-ID3 cells was determined by in vivo subcutaneous transplantation (n = 6). Tumor volumes are expressed as mean ± SD. Inset: representative images of tumors derived from Ast^{Ink4a/Arf-/-}-control and Ast^{Ink4a/Arf-/-}-ID3 cells. *P<0.05.

(C) Left panel: Number of neurospheres (>10 μm) in Ast^{Ink4a/Arf-/-}-control and Ast^{Ink4a/Arf-/-}-ID3 cells (400 cells/12-well plate) grown for 14 days in serum-free medium supplemented with EGF
and bFGF. **P<0.01. Immunofluorescence images (magnification, 100×; scale bar = 50 μm) showing various stem cell and differentiated cell markers in cryomicrodissected neurospheres derived from AstInk4a/Arf−/−-ID3 cells (right). Right panel: Tumorigenic potential of AstInk4a/Arf−/−-control and AstInk4a/Arf−/−-ID3 cells was determined by in vivo subcutaneous transplantation (n = 6). Tumor volumes: mean ± SD. Representative photos (Inset; scale bar = 2 mm) showing tumors derived from AstInk4a/Arf−/−-control and AstInk4a/Arf−/−-ID3 cells.

(D) Immunohistochemical detection of ID3+, CD133+, Nestin+, S100β+, O4+, and CD31+ cells in tumors derived from AstInk4a/Arf−/−-control and AstInk4a/Arf−/−-ID3 cells. Scale bar = 50 μm.

**Fig. 4. EGFR-ID3 signaling induces cytokines that promote endothelial tube and neurosphere formation.**

(A) Left panel: U87MG-ID3 cells show increased GRO1, IL6, and IL8 secretion as determined by the angiogenesis antibody array. Right panel: High levels of GRO1, IL6, and IL8 mRNA in the U87MG-ID3 cells were confirmed by real-time PCR analysis. Data are expressed as mean ± SD. **P<0.01.

(B) In vitro tube formation was elevated in HUVECs exposed to conditioned medium from U87MG-GRO1, U87MG-IL6, U87MG-IL8, and U87MG-ID3. The knockdown of GRO1, IL6, and IL8 in U87MG-ID3 cells suppressed ID3-mediated induction of tube formation. a,b,c,d,e,f,g,h P < 0.05; A,B,C,D,E,F,G,H P < 0.01.

(C) Neurosphere formation assay. EGFRvIII, ID3, IL6, and IL8 increased neurosphere formation of U87MG cells. A,B,C,D,E,F,G,H P < 0.01.
Fig. 5. Role of ID3 and ID3-regulated cytokines in Notch signaling and GSC marker expression.

(A) Real-time RT-PCR analysis revealed differential expression of Notch signaling-related genes (JAG1, HES1, and HEY1) and NSC markers (CD133 and Nestin) in the U87MG-Control, 87MG-ID3, U87MG-GRO1, U87MG-IL6, and U87MG-IL8 cells. \(^{a,b,c,d,e} P < 0.05; ^{A,B,C,D,E} P < 0.01.\)

(B) shRNA-mediated knockdown of ID3 in XO1 and XO2 GSC lines suppressed expression of GSC markers (CD133 and Nestin), cytokines (IL6 and IL8), and Notch signaling-related genes (Jagged1, Hes1, and Hey1) as assessed by real-time RT-PCR. \(*P<0.05; **P<0.01.\)

Fig. 6. EGFR-ID3-IL6 signaling gives rise to tumors with cellular heterogeneity.

(A) Representative images (upper panel; scale bar = 2 mm) and weight (lower panel) of tumors obtained from nude mice (n = 6) injected subcutaneously with U87MG-Control, U87MG-ID3, U87MG-EGFRvIII, U87MG-EGFRvIII-shID3, U87MG-IL6, and U87-ID3-shIL6 cells. \(^{a,b,c,d,e,f} P < 0.05; ^{A,B,C,D,E,F} P < 0.01.\)

(B) Immunofluorescence images (200×; scale bar = 50 µm) of CD15\(^+\) NSCs (red) or NG2\(^+\) oligodendrocytes (red); Nestin\(^+\) NSCs (red) or Tuj1\(^+\) neurons (red); S100\(\beta\)^+ astrocytes (red) in the tumors described in (A). Representative immunohistochemistry images showing CD31\(^+\) endothelial cells in these tumors.

(C) Kaplan-Maler survival rates of nude mice orthotopically injected with U87MG-Control, U87MG-ID3, U87MG-EGFRvIII, U87MG-EGFRvIII-shID3, U87MG-IL6, and U87-ID3-shIL6 cells.
Fig. 7. Effect of EGFR inhibitors in GSCs with activated EGFR-ID3 signaling.

(A) The EGFR inhibitor AG1478 (10 μM for 12 hrs) markedly reduced levels of ID3, p-AKT, and p-Smad1,5,8 in EGFRvIII-expressing X01 and X03 cells, but 12 hrs after withdrawal, ID3, p-AKT, and p-Smad1,5,8 were restored.

(B) Recovery of neurosphere formation in X01, X03 and U87MG-EGFRvIII glioma cells by withdrawal of EGFR inhibitors. Cells were treated with AG1478 (1 μM) and Gefitinib (1 μM) for 3 days, and then cells were allowed to grow for 12 days without AG1478 and Gefitinib.

a,b,c,d,e,f P < 0.05; A,B,C,D,E,F P < 0.01.

(C) EGFR signaling leads to acquisition of GSC characteristics and angiogenesis by induction of ID3 via the AKT-dependent activation of Smad5, and subsequent induction of ID3-regulated cytokines signaling.
Figure A shows the cell number over five days in culture for Ast Ink4a/Arf−/− cells treated with 10% or 0.5% FBS, with ID3 significantly increasing cell proliferation compared to Puro treatment. Western blot analysis indicates the expression of ID3, pRb, Cdk4, Cdk2, Cyclin D1, Cyclin E, and Cyclin A, with a-tubulin serving as a loading control.

Figure B presents a bar graph showing the foci number for Puro and ID3 treatments in Ast Ink4a/Arf−/− cells, with the ID3 treatment resulting in a significantly higher foci number compared to Puro.

Figure C illustrates neurosphere formation in Puro and ID3 treatments in Ast Ink4a/Arf−/− cells, with a significant reduction in neurosphere formation in the ID3 treatment group. Immunofluorescence images show a decrease in DAPI/CD133/S100β and Nestin/Tuj1 expression, while an increase in DAPI/O4/Sox2 expression is observed.

Figure D displays immunohistochemistry results forAst Ink4a/Arf−/− cells treated with Puro or ID3, with staining for CD133, Nestin, S100β, O4, and CD31, highlighting the differences in expression patterns between the two treatments.
EGFR-AKT-SMAD signaling promotes formation of glioma stem-like cells and tumor angiogenesis by ID3-driven cytokine induction

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